In vivo Modulation of Avidity in Highly Sensitive CD8⁺ Effector T Cells Following Viral Infection

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Abstract

Numerous studies have demonstrated a critical role for T cell avidity in predicting *in vivo* efficacy. Even though the measurement of avidity is now a routine assessment for the analysis of effector and memory T cell populations, our understanding of how this property is controlled *in vivo* at both the population and individual cell levels is limited. Our previous studies have identified high avidity as a property of the initial effector population generated in mice following respiratory virus infection. As the response progresses, lower avidity cells appear in the effector pool. The studies described here investigate the mechanistic basis of this *in vivo* regulation of avidity. We present data supporting *in vivo* avidity modulation within the early high avidity responders that results in a population of lower avidity effector cells. Changes in avidity were correlated with decreased lck expression and increased sensitivity to lck inhibitors in effector cells present at late versus early times postinfection. The possibility of tuning within select individual effectors is a previously unappreciated mechanism for the control of avidity *in vivo*.

Introduction

IT IS CLEAR FROM A NUMBER OF STUDIES that not all antigen-specific cytotoxic T lymphocytes (CTL) are equally effective *in vivo*. As one example, differences in efficacy can arise from differences in poly-functionality of effector populations (5). Another critical attribute that determines *in vivo* efficacy is functional avidity. Avidity is defined by the amount of antigenic peptide required for CD8⁺ T cell activation or effector function, with high avidity cells exhibiting greatly increased sensitivity to peptide-major histocompatibility complex (pMHC). The initial report in this regard demonstrated that adoptively transferred high avidity CTL can reduce viral burden by 1000-fold following infection with vaccinia virus, while low avidity CTL are ineffective at viral clearance (3). In the case of viral infections, the increased efficacy of high avidity CTL results, at least in part, from their ability to recognize virus-infected cells very early after infection (22). The crucial involvement of high avidity CTL in the clearance of viruses and tumors has been confirmed in a number of studies (5,6,28,34,43,55,61,63). That said, there may be circumstances wherein lower avidity cells play a critical role. For example, cells exhibiting reduced avidity may be protected from exhaustion in models of chronic antigen exposure (15) and may play important roles in the clearance of some tumor cells (54,57,58).

At the individual cell level, several factors have been implicated in influencing functional avidity, including TCR af-

finity and the absolute level or isoform of CD8 expressed (16,18,61). The contribution of TCR affinity to functional avidity has been studied largely through utilization of tetramer dissociation assays as a correlate of TCR affinity (13,61). It is worth noting that this approach limits the interpretation to some degree as tetramer dissociation can be influenced by a number of factors other than TCR affinity, for example, TCR membrane organization (23,25) and the contribution of CD8 (21,41). Further, tetramer dissociation is not always predictive of functional avidity (1,12,24,40,47,52). Nonetheless, these data are consistent with the notion that the affinity of the TCR can contribute to functional avidity, and in some cases differential V β usage has been shown to correlate with differences in peptide sensitivity (35). Finally, changes in localization or level of molecules involved in TCR signal transduction have the potential to impact the requirement for peptide antigen. Increases in the level of lymphocyte specific tyrosine kinase (lck) (56), ZAP-70 (32,49) are associated with increased peptide sensitivity, while increases in the SHP-1 phosphatase appear to decrease peptide sensitivity (32).

We have previously assessed the evolution of avidity following respiratory infection with vaccinia virus (VACV) and parainfluenza virus 5 (PIV5). In these analyses, we found that the initial responding effector cells in the lung-draining mediastinal lymph nodes (MLN) were of exceptionally high avidity (30). As the response increased in size over the next several days, the population as a whole exhibited lower avidity compared to the initial responders.

These data suggested that there was either delayed recruitment of low avidity effectors into the responding population, or that high avidity cells present at early times were undergoing avidity modulation towards a low avidity phenotype. In support of the potential for modulation in effectors, we have found in *in vitro* models that an individual cell can give rise to both high and low avidity progeny (36).

The studies presented here test the hypothesis that the lower avidity cells present at the peak of the response following respiratory infection are derived from the high avidity cells present at early times. We find that, while in the absence of selective pressure these *in vivo* generated effector cells exhibit high avidity, they possess the potential to acquire a lower avidity phenotype during *in vivo* differentiation and do so over the course of viral infection. Interestingly, the decreased avidity present in effectors at later times was associated with decreased lck expression and increased sensitivity in lck inhibitors, suggesting active regulation of lck may contribute to the modulation of avidity observed *in vivo*.

Materials and Methods

Mice and viruses

Female BALB/c mice (6–8 weeks of age) were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Thy1.1⁺BALB/c mice were originally purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred within our animal facility. All research performed on mice in this study complied with federal and institutional guidelines set forth by the Wake Forest University Animal Care and Use Committee. All studies were approved by the Wake Forest University Animal Care and Use Committee.

P815 is a DBA/2-derived (H-2^d) mastocytoma. Recombinant WT parainfluenza virus 5 (PIV5) was constructed as previously described (29).

Infection with PIV5

Mice were anesthetized with Avertin (2,2,2-tribromoethanol) by intraperitoneal (i.p.) injection. Virus (10⁶ PFU for naïve or 10⁷ PFU for memory animals) was administered intratracheally in 50 μ L of PBS. Mice were considered at the memory time point 40 days post infection.

In vivo treatment with anti-CD62L antibody

Mice received 10⁶ PFU of PIV5 by intratracheal instillation. Eighteen hours following infection, mice were treated with 100 μ g of LEAF purified anti-mouse CD62L (MEL-14) or LEAF purified Rat IgG2a isotype by i.v injection (BD Biosciences, San Jose, CA). On d7 postinfection, MLN were isolated for analysis.

Isolation of activated/virus-specific populations

At the designated days post secondary infection (D1 and/ or D6), mediastinal lymph nodes (MLN) were removed and pooled from multiple mice. Cells were labeled with CD8 α -PerCPCy5.5 and CD25-APC (D1 post 2^o challenge) or CD8 α -PerCPCy5.5 and M_{285–293}/L^d tetramer APC (provided by National Institutes of Health (NIH) Tetramer Core Facility at Emory University in Atlanta, GA) (D6 post 2^o challenge) and sorted using a FACSAria cell sorter running BD Biosciences

DIVA software. Where indicated, cells were also labeled with 5(6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Invitrogen, Carlsbad, CA) at a concentration of 2.5 μ M. Purity of the CD8⁺CD25⁺, CD8⁺CD25[–], and CD8⁺M_{285–293}/L^d tetramer⁺ populations was >93% in all experiments.

Expansion of sorted populations in vitro

For nonantigen specific expansion, cells were cultured for 3 days in a 24-well plate containing 2 mL RPMI-1640 media supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1x nonessential amino acids (NEAA), 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M 2-ME, 10% FBS, 10% T-stim (BD Biosciences), 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO), and 500 ng/mL ionomycin (Sigma-Aldrich). For antigen-specific expansion, sorted populations were cultured with 10^{–5}M or 10^{–10}M M_{285–293} (IPKSAKLFF) peptide pulsed irradiated BALB/c splenocytes. Cultures were restimulated weekly.

Avidity analysis

Cells were incubated in the presence of GolgiPlug (BD Biosciences) and titrated concentrations of M_{285–293} peptide for 5 hours. Following stimulation, cells were stained with antibodies to detect cell surface antigens as indicated, followed by fixation and permeabilization using Cytofix/Cytoperm (BD Biosciences). Cells were subsequently stained with anti-IFN γ antibody. Samples were fixed in 2% PFA and acquired on a BD Biosciences FACSCalibur flow cytometer using CellQuest software.

V β usage analysis

Three or 7 days post *in vitro* stimulation, cells were stained with CD8 α -PerCPCy5.5, M_{285–293}/L^d tetramer-APC and V β antibodies conjugated to FITC (TCR Screening Panel) using those antibodies that detect V β regions that are present in BALB/c (V β 2, 4, 6, 7, 8.1/8.2, 8.3, 9, 10^b, 13, 14, 17^a).

CDR3 spectratyping

CDR3 spectratyping was performed by BioMed Immunotech (Tampa, FL). In brief, total RNA was isolated from sorted CD8⁺tetramer⁺ populations. RNA was subjected to combined cDNA synthesis and one-step PCR amplification, followed by nested PCR amplification using 5' V β 8.1 and 8.2 primers and a 3' C β primer. CDR3 analysis was carried out at BioMed Immunotech.

Adoptive transfer of d1 CD8⁺CD25⁺ sorted cells into infected mice

A sorted population of CD8⁺CD25⁺ (6 $\times$ 10⁴ cells) was intravenously transferred into BALB/c Thy1.1⁺ recipient mice that were day 1 post PIV5 infection. The purity of the transferred population was $\geq$ 93%. Seven days post transfer, the lung and MLN were harvested. Lung lymphocytes were isolated by digestion of tissue with Collagenase D (Roche Diagnostics, Indianapolis, IN) and a Histopaque-1083 gradient (Sigma-Aldrich). Analysis of cytokine was performed as described above. Transferred cells were detected by inclusion of Thy1.1 antibody, and endogenous cells by Thy1.2 antibody.

Analysis of lck protein level and effect of lck inhibitors

At designated days post-secondary infection (d2–d6), mediastinal lymph nodes (MLN) were removed and pooled from multiple mice. Cells were labeled with anti-CD8 α , anti-CD25, anti-CD44, and M_{285–293}/L^d tetramer. Labeling with anti-CD4 and CD19 were used as a negative gating strategy to improve detection of tetramer⁺ cells. Following surface staining, cells were fixed and permeabilized with Cytofix/CytoPerm. PE conjugated anti-lck antibody (BD Biosciences) was used to detect lck. For analysis of the sensitivity to lck inhibitors, cells were incubated with tetramer to both stimulate production of IFN γ and mark antigen-specific cells. This approach was chosen as tetramer staining post-peptide stimulation is suboptimal as a result of TCR internalization. Titrated concentrations of the Src kinase inhibitors PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine) or 7C-PP (cyclopentyl-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine) were added during the 5 h culture period. Following stimulation, cells were stained with antibodies to detect cell surface antigens together with tetramer as indicated, followed by fixation and permeabilization using Cytofix/CytoPerm (BD Biosciences) and subsequent staining with anti-IFN γ antibody.

Quantitation of lck mRNA levels by real-time PCR

For lck mRNA analysis, a commercially available TaqMan primer-probe set specific for lck was used. Lymphocyte RNA was extracted using a standard Trizol (Invitrogen, Grand Island, NY) phenol/chloroform extraction. cDNA was synthesized from mRNA by reverse transcription using SuperScript III RT kit (Invitrogen) and random primers (Invitrogen). RT-PCR (qRT-PCR) was performed using the Applied Biosystems 7500 real-time PCR system. Raw data values were normalized to GAPDH mRNA levels. Fold change was calculated using the $\Delta\Delta C_t$ method.

Results

Lower avidity cells present at later times are not the result of suboptimal activation of late emigrants into the MLN

Our previous studies demonstrated that the earliest responding CD8⁺ T cells following both primary and sec-

ondary respiratory infection are predominantly of high avidity (30). As the response continues, the population exhibits a mixed avidity phenotype (30), suggesting that cells with lower avidity were either recruited into the response at later times or were high avidity cells that underwent active modulation of their peptide sensitivity. It was previously reported that cells that are late recruits into the anti-viral immune response can exhibit reduced proliferation and altered function (9,20,26,44). Based on this, we postulated that the lower avidity cells that we observed could result from cells that enter the LN at later times postinfection, a time-point at which the APC/environment present in the MLN has altered such that cells with decreased peptide sensitivity can be activated.

To address the possibility that low avidity cells were later immigrants into the lymph node, we treated mice with anti-CD62L antibody or an isotype control antibody 18 h following infection with PIV5. Treatment with anti-CD62L antibody has been previously shown to abrogate further entry of naïve T cells into the LN (62). On d7 p.i., a timepoint at which both high and low avidity cells are present in unmanipulated animals, the effector cell population present in the MLN was assessed. The data in Figure 1 show that the number of effector cells specific for the immunodominant M_{285–293} epitope was significantly decreased in animals treated with anti-CD62L antibody. This finding is consistent with the failure to recruit the full repertoire of precursor cells specific for the epitope when anti-CD62L antibody is administered. However, analysis of the functional avidity of the effectors generated from anti-CD62L-treated versus isotype-treated animals revealed similar sensitivities of these two populations to peptide antigen (Fig. 1B). These data suggest the lower avidity cells present at later times in the generation of the immune response are not the result of a population of cells that is recruited into the response with delayed kinetics because of late entry into the lymph node.

CD25 selection enriches for M-specific cells that are activated at early times postinfection

Given the above finding together with our *in vitro* studies demonstrating the ability of an individual cell to actively modulate avidity in response to the stimulatory conditions encountered (36), we tested the possibility that a portion of the early high avidity population underwent avidity

FIG. 1. Effectors generated in mice treated with anti-CD62L antibody are reduced in number, but similar in functional avidity compared to control mice. Mice received 10^6 PFU of PIV5 by intratracheal instillation. Eighteen hours after infection, mice were treated with 100 μ g of anti-mouse CD62L or purified Rat IgG2a isotype. On d7 postinfection, MLN were isolated for analysis. **(A)** Average number of B8R-specific cells from 5 mice treated with anti-CD62L or isotype control antibody. Statistical analyses were performed using Student's *t* test. ***p* = 0.002. **(B)** Avidity of effector cells generated from animals treated with anti-CD62L or isotype control antibody. Data are representative of three independent experiments.

modulation. If this were the case, there should be a precursor-product relationship between the early high avidity and later mixed avidity populations.

In order to address this question, we developed an approach to isolate the initial population of responding virus-specific cells. Given that the number of precursors responding following primary infection is extremely limited, together with the observation that high avidity cells are the initial responders post-secondary infection (similar to what is observed in the primary response) (30), we chose to develop this model in the context of secondary infection where the responder number was adequate for experimental examination. BALB/c mice were challenged with PIV5 on d40 post primary infection. On d1 post secondary infection, we isolated cells from the MLN of infected mice and stained them for the presence of CD25⁺ cells. At this time, approximately 1.6% of CD8⁺ cells expressed this activation marker (Fig.

2A). CD25⁺CD8⁺ and CD25⁻CD8⁺ populations were sorted and subsequently cultured for 3 days in the presence of PMA and ionomycin to nonspecifically expand the cells for further analysis. Cells were then stained with M₂₈₅₋₂₉₃/L^d tetramer and CD8 to assess the presence of virus-specific cells in the cultured populations. These analyses demonstrated that the selection for CD25⁺ cells present in the MLN on d1 postinfection resulted in a population that was enriched for PIV5 M-specific cells (Fig. 2B), supporting this as a valid approach for isolation of early responding virus-specific cells.

As we were developing this approach to determine the relationship between the early and late responder population, it was important to ensure that the stimulation we were using did not result in the preferential expansion of a subset of M-specific clones. To assess this possibility, we labeled isolated MLN cells with CFSE prior to sorting. As above, CD25⁺ populations were sorted from mice on d1 post-

FIG. 2. Isolation of CD25⁺ cells at d1 post-secondary infection enriches for early responders to PIV5. BALB/c mice infected with PIV5 >40d prior were secondarily infected. On d1 postinfection, MLN were isolated and pooled. **(A)** Following staining, CD25⁺CD8⁺ and CD25⁻CD8⁺ populations isolated by sorting. **(B)** Sorted cells were cultured in the presence of 50 ng/mL PMA and 500 ng/mL ionomycin for 3 days, following which cells were stained with M₂₈₅₋₂₉₃/L^d tetramer and CD8 to assess the presence of virus-specific cells in the cultured populations. **(C)** MLN cells from mice that were d1 post secondary infection were labeled with CFSE and CD25⁺CD8⁺ cells sorted. Cells were cultured in the presence of PMA and ionomycin for 3 days, following which they were stained with M₂₈₅₋₂₉₃/L^d tetramer and CD8. Unstimulated CFSE-labeled MLN cells from which the CD25⁺ population was obtained is shown as a control. These data are representative of three independent experiments each with 10 animals.

secondary infection and subsequently stimulated with PMA and ionomycin. Following the 3-day culture period, we determined the level of CFSE in the M-specific population. The data in Figure 2C show that the $M_{285-293}/L^d$ tetramer⁺ population was relatively homogeneous in CFSE intensity. Thus, this approach results in similar expansion of the M-specific cells and as such the population present at the end of the culture period should reflect that present at d1 postinfection.

Early and late M-specific cells exhibit similar V β usage

Having established our approach for isolation of the early responders and nonbiased expansion, we next determined whether the early and late responder populations shared their TCR receptor repertoire. As a first step, we analyzed the V β regions utilized by the early high avidity (d1) and late mixed avidity (d6) M-specific populations. BALB/c mice that had been infected with PIV5 40 days prior were challenged with PIV5 and MLN cells isolated on d1 or d6 following infection. For mice that were d1 post-challenge, MLN cells were isolated based on CD8 and CD25 and expression. For animals that were d6 post-challenge, MLN cells were sorted based on the expression of CD8 and $M_{285-293}/L^d$ tetramer. (As CD25 is a transient marker of activation, it could not be used at this timepoint, as a large portion of tetramer⁺ cells had lost high expression of CD25). Isolated cells were expanded in the presence of PMA and ionomycin for 3 days, followed by staining with CD8, tetramer, and a panel of V β -specific antibodies (Fig. 3A). Again, our preliminary experiments supported this approach as nonbiasing for expansion. For both the d1 and d6 populations, we found that V β 8.1/8.2 was the predominant region utilized, with approximately 84% (d1) and 92% (d6) of tetramer⁺ cells on average staining positive for this antibody. V β 8.3 was the next highly utilized with approximately 10% (d1) and 3% (d6) of cells staining positive. Thus, 94% (d1) and 95% (d6) of tetramer cells utilized V β 8. As further evidence that our expansion approach resulted in a population of cells that was reflective of the initially isolated populations, we performed V β analysis on the d6 population directly ex vivo. This analysis revealed a similar V β distribution in expanded versus nonexpanded populations (data not shown). Together, these data show that there was no distinct V β usage by the high avidity population present at early times postinfection versus the mixed avidity population present at the peak of the response.

Early and late M-specific cells exhibit similar spectratype profiles

To examine the TCR diversity in more detail, we performed spectratype analysis on the expanded early and late populations. MLN cells were isolated from secondarily infected animals as described above (CD25⁺CD8⁺ from d1 animals and CD8⁺tetramer⁺ cells from d6 animals). Sorted cells were expanded with PMA and ionomycin for 6 days. At the end of the culture period, tetramer⁺ cells were sorted from each population (93% pure). While d6 cultures had already been subjected to isolation based on tetramer staining, we wanted to use a consistent approach across the two cultures. As a control we analyzed splenocytes from naïve mice, which showed the expected normally distributed population (Fig. 3B). Analysis of d1 cultures revealed a non-Gaussian

FIG. 3. The early and late responding cells exhibit a similar TCR usage profile. **(A)** MLN from mice that were d1 or d6 post secondary PIV5 infection were harvested and pooled. CD25⁺CD8⁺ cells were sorted from animals that were d1 post-secondary infection and CD8⁺ $M_{285-293}/L^d$ tetramer⁺ cells sorted from animals that were d6 post-secondary infection. Sorted cells were restimulated with PMA and ionomycin for 3 days. Cultures were then stained with $M_{285-293}/L^d$ tetramer, anti-CD8 antibody and a panel of V β -specific antibodies. These data are representative of two independent experiments each containing >10 mice for d1 analysis and 5 mice or d6 analysis. **(B)** For CDR3 analysis, cells were sorted as above and populations expanded with PMA and ionomycin for 6 days. CD8⁺ $M_{285-293}/L^d$ tetramer⁺ cells in the cultures were then isolated by sorting and subjected to spectratype analysis. Naïve splenocytes served as a control for normally distributed CDR3 lengths. Results reflect cells from 27 mice pooled from two experiments for d1 populations and 8 mice pooled from two experiments for d6. Pooling was necessary to obtain adequate RNA for the analysis.

distribution with regard to CDR3 length, suggesting an oligoclonal response (Fig. 3B). When the analysis of d6 cultures was compared to the d1, a similar pattern was observed. We did note the presence of a minor population within the d1 V β 8.2 population that was absent at d6, suggesting this subpopulation may not be retained throughout the *in vivo* expansion phase. The loss of this minor population should not account for the significant reduction in avidity in the late response, as presumably most cells at this early time were of high avidity and thus loss of one subpopulation should not reduce avidity. Instead, if the lower avidity nature of the late population reflected the presence of new clones with a lower affinity TCR, we would have expected the emergence of novel populations in the effectors present at late times. These data suggest that with regard to both V β usage and CDR3 length, the early and late

populations exhibit similarity. These results demonstrate the initial responding population that is almost exclusively high avidity cannot be distinguished from the later population, which contains a mixed avidity population, based on either V β usage or CDR3 length.

In the absence of selective pressure, early responders display high avidity

The similarity in the TCR repertoire observed in the above studies is consistent with a model wherein the high avidity cells could be the progenitors of the lower avidity cells present at later times. If this were the case, one would expect that the early high avidity cells could give rise to lower avidity cells following culture *ex vivo*. In this model, the acquisition of low avidity may be a programmed event that occurs during the process of differentiation or alternatively may require active selective pressure.

We first determined whether T cells present at early times maintained their high avidity phenotype following nonselective short-term expansion. Cells isolated from the MLN of memory mice on d1 post challenge were sorted based on CD8⁺CD25⁺ expression, and subsequently cultured in the presence of PMA and ionomycin. Following 3 days of culture, the peptide sensitivity of the cells was determined by stimulation in the presence of titrated concentrations of M₂₈₅₋₂₉₃ peptide. We chose this timeframe as low avidity cells appeared in the responding effector population within a 3-day period *in vivo* (30). Thus, if down-modulation of avidity were programmed, we reasoned that we should see a loss of avidity over this period in culture. For comparison with a population of overall lower avidity, we utilized MLN cells from mice that were d6 post-secondary challenge. The data in Figure 4A show that the cells expanded from mice d1 post-challenge were of significantly higher avidity (approximately 100-fold) compared to those from mice that were d6 post challenge. These data confirm the high avidity nature of these initially responding cells and shows that these cells are not programmed to decrease peptide sensitivity.

*High avidity cells present at early times can give rise to low avidity effectors after *in vitro* stimulation with modulatory concentrations of peptide*

While the above results suggested that the default differentiation pathway for the early effectors was development into high avidity responders, we sought to determine whether these cells could give rise to low avidity effectors under selective pressure. Previous studies from our laboratory have established the ability of individual cells to modulate avidity during the initial encounters with antigen (36). Here we tested the hypothesis that these early effectors could give rise to low avidity cells following stimulation with APC bearing high levels of pMHC, a well-established approach for generating low avidity cells *in vitro* (16–18,36–38). MLN cells were isolated from memory mice on d1 post-secondary infection with PIV5 and CD25⁺CD8⁺ cells sorted. In parallel, tetramer⁺CD8⁺ MLN cells were isolated from mice that were d6 post-secondary infection as a source of a population known to contain both high and low avidity cells (30). Isolated cells were then cultured in the presence of APC that had been

FIG. 4. Early responders exhibit high avidity but can differentiate into low avidity cells under selective pressure. **(A)** MLN were isolated on d1 post-secondary infection. CD25⁺CD8⁺ cells were sorted and restimulated with PMA and ionomycin for 3 days. At this time, cells were restimulated with titrated concentrations of M₂₈₅₋₂₉₃ peptide and the production of IFN γ assessed by ICCS. As an indicator of the peptide sensitivity of a mixed avidity population, MLN cells from mice d6 post-secondary infection were used directly *ex vivo*. Percent maximal IFN γ production is shown given that not all CD8⁺ cells present at this time in the two cultures are M-specific. Averaged data from pooled animals in 2 (d6) or 3 (d1) experiments are shown. **(B)** MLN from secondarily infected memory mice were isolated on d1 or d6 p.i. CD25⁺CD8⁺ cells were sorted from d1 animals and tetramer⁺CD8⁺ cells from d6 animals. Isolated populations were divided and stimulated with splenocytes pulsed with either 10⁻⁵ or 10⁻¹⁰ M peptide. Cultures were restimulated weekly. Tertiary cultures were incubated with titrated concentrations of M₂₈₅₋₂₉₃ peptide and the production of IFN γ assessed by ICCS. These data are representative of three independent experiments, each using cells sorted from 10 pooled animals as the source population for line generation.

previously pulsed with a high (10⁻⁵M) or low (10⁻¹⁰M) concentration of the M₂₈₅₋₂₉₃ peptide to expand cells for analysis. Both high and low avidity cells could be generated from the d1 population and was dictated by the amount of peptide antigen utilized (i.e., cells stimulated in the presence of high pMHC required higher amounts of peptide to elicit effector function and were thus of lower avidity compared to those generated by stimulation with low pMHC) (Fig. 4B). Further, these high and low avidity populations exhibited avidity similar to their counterpart culture generated from the d6 animals. Cell recovery from the d1 mice was similar in the cultures stimulated with the two peptide concentrations, suggesting that we were not selecting for a minor subpopulation of low avidity cells that was selectively expanded *in vitro*. These data strongly support the ability of high avidity early effectors to give rise to low avidity cells.

*High avidity cells present at early times can give rise to a population of effectors *in vivo* that possess similar avidity to that of effectors generated from the total endogenous repertoire*

The previous study demonstrated that the high avidity cells present at the initiation of the response were capable of giving rise to lower avidity cells. Thus, we determined whether the early responding high avidity population gave rise to low avidity effectors *in vivo*. CD25⁺CD8⁺ cells were isolated from the pooled MLN cells of animals that were d1 post-secondary infection as described above. Based on the purity of the sort, the potential contamination with nonactivated (CD25⁻) M₂₈₅₋₂₉₃-specific cells is <0.5%. The sorted population was transferred into Thy1.1 recipient animals that were d1 post PIV5 infection. This approach allowed for differentiation of the transferred cells under conditions that would normally be present during the continued expansion of these cells in their original host. On d8 postinfection (d7 post-transfer), MLN and lung tissues were isolated. Cells from these tissues were stimulated in the presence of titrated concentrations of M₂₈₅₋₂₉₃ peptide. IFN γ production in Thy1.1⁺ (endogenous) and Thy1.2⁺ (adoptively transferred) populations was assessed by ICCS. Figure 5 shows that the dose response curves for the population generated from the transferred early responder high avidity cells was similar to that for the endogenous population. This result supports the model that high avidity early responders can give rise to both high and low avidity effector cells *in vivo* during the normal differentiation process that occurs as the anti-viral effector population is generated.

Early effectors enriched for high avidity have increased levels of lck compared to the mixed avidity population present at later times postinfection

One mechanism that has been reported as a modulator of peptide sensitivity is the regulated expression of lck (56), a critical mediator of ζ chain phosphorylation (39,46). Thus, we tested the possibility that modulation of avidity was associated with changes in lck levels in effector cells present in the MLN at early vs. late times p.i. MLN were isolated post-secondary infection and M₂₈₅₋₂₉₃-specific cells identified by tetramer and CD8 staining. We assessed lck levels in CD25⁺tetramer⁺ cells at d2 and d6 postinfection. Lck levels in the total CD8⁺ populations for both timepoints are shown for comparison. Of note, d2 data are shown as tetramer staining in d1 cells was challenging to detect in the CD25⁺ population at this time, likely the result of TCR internalization following activation.

CD25⁺tetramer⁺ cells present at d5-6 postinfection had, on average, a 29% decrease in the amount of lck present compared to that in cells from d2 animals (Fig. 6A and B). Gating on the total tetramer⁺ population revealed even lower levels of lck (data not shown). However, we restricted our analysis to CD25⁺ populations to control for any potential differences in lck associated with recent antigen encounter. Further, in agreement with the d2 findings, activated CD8⁺ cells present at d1 (as measured by CD25 expression) did exhibit high levels of lck similar to the d2 CD25⁺tetramer⁺ population, supporting the increased expression of lck in the earliest responding cells (data not shown).

To determine whether the reduced lck protein observed in the late effectors was associated with decreased mRNA,

FIG. 5. High avidity early responders give rise to an effector population that exhibits an avidity profile similar to that of the total endogenous responder population. MLN from secondarily infected Thy1.2⁺ mice were isolated on d1 p.i. Pooled cells from 12 animals were stained and CD25⁺CD8⁺ cells sorted. 5×10^4 sorted cells were then transferred i.v. into Thy1.1⁺ recipients that had been infected with PIV5 1 day prior. On day 7 post-transfer, MLN and lung cells were isolated from the recipient animals. The avidity of the transferred and endogenous M₂₈₅₋₂₉₃-specific cells was determined by the production of IFN γ in response to stimulation with titrated concentrations of peptide. These data are the mean $\pm$ SEM of 4 recipient animals assessed in two independent experiments.

d2 and d6 effectors were sorted and lck message assessed by real-time PCR. The data in Figure 6C show that the level of mRNA in d6 cells was modestly higher (approximately 2-fold) compared to d2 cells, counter to what we observed at the protein level. These data suggest that the difference in lck protein present in the early versus late effectors is controlled at a step downstream of transcription and mRNA turnover.

Based on the decreased expression of lck in d6 cells, we predicted that these effectors would exhibit increased sensitivity to inhibition of lck activation. To test this possibility, cells from animals that were d2 or d6 postinfection were treated with titrated concentrations of the Src kinase inhibitor, 7C-PP (cyclopentyl-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine) (8,11,14). Increased susceptibility to these inhibitors has been previously reported to correlate with low functional avidity (51). The data in Figure 6D (top panel) show that the lower avidity population at d6 was more sensitive to inhibition by 7C-PP compared to d2 cells, in agreement with their decreased avidity. To confirm these results, we tested a second Src kinase inhibitor, PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine) (31,33). Again, d6 effectors were more sensitive to inhibition compared to their d2 counterpart. Together these data support a model wherein lck protein levels are actively regulated in effector cells over the course of the response. Furthermore, the differences in lck are associated with changes in functional avidity.

FIG. 6. Initial responders exhibit increased levels of lck compared to late effectors. On day 2 or 5/6 post-secondary infection, MLN cells were stained with M_{285–293}- tetramer together with antibodies to CD8, CD44, CD25, and lck. The level of lck present in activated CD44^{hi}CD25⁺CD8⁺tetramer⁺ cells is shown. Representative data are shown in **A** and averaged data from 6–9 animals in **B**. Data in **B** represent the fold change in MFI compared to the levels found in d2 cells. The MFI of d2 cells was assigned a value of 1 and the relative fold decrease was then calculated for d6 populations. Statistical analyses were performed using Student's *t*-test. ***p* value < 0.0001. **(C)** Effector cells were sorted on d2 (CD8⁺CD25⁺tetramer⁺) or d6 (CD8⁺tetramer⁺) post-secondary infection. Expression values were calculated relative to GAPDH as an internal standard (*top panel*) and fold changes were calculated using the $\Delta\Delta C_t$ method (*bottom panel*). **(D)** MLN cells isolated at d2 or d6 post secondary infection were treated with titrated concentrations of PP2 or 7CCP during stimulation with peptide antigen. IFN γ was measured in CD8⁺CD44^{hi}tetramer⁺ cells (d6) or CD8⁺CD25^{hi}CD44^{hi}tetramer⁺ cells (d2). Averaged data with SEM obtained from two experiments, each containing 3 mice for d2 and 2 mice for d6, are shown.

Discussion

The functional avidity of responding CD8⁺ effector T cells plays a critical role in determining the efficacy of these cells *in vivo*, with high avidity an indicator of cells with increased viral clearance capability (3,5,6,28,43,55,61,63). In our previous studies, we found that the avidity of the effector population present at the peak of the response following respiratory infection is not reflective of the initial population of responding cells, with the mature effector population exhibiting significantly low avidity (30). Changes over time in the avidity of an effector population has been observed in other systems, although whether avidity increases or decreases appears to be epitope dependent (27,30,51,56). Here we have explored the basis for the loss in avidity observed in our system. The studies presented here support a model wherein changes in peptide sensitivity observed at the population level can occur as a result of avidity modulation within individual cells. Our data support a model where at least a portion of the low avidity cells present following respiratory infection are the result of decreases in avidity within a subset of the high avidity clones present within the initial responding population. Importantly, in our model, modulation occurred within the context of an endogenous repertoire of responding cells that was not biased by the presence of a monoclonal TCR. These data suggest the change in avidity that occurs between the initiation and peak of the response *in vivo* is an active process. Thus down-modulation of avidity appears not to be programmed into the cells at the time of initial activation.

We propose that cells encounter modulatory signals subsequent to activation that result in changes in peptide sensitivity. The differentiation of T cells by signals temporally distinct from those received during initial activation has been reported (10,42,48). For example, in a study from Harty and colleagues, T cells primed as a result of DC vaccination preferentially acquired a memory phenotype (10). However, if these cells were exposed to inflammatory mediators subsequent to activation, they developed into short-lived effector cells (48). Further, in the case of CD4⁺ T cells, numerous studies have demonstrated plasticity with regard to movement between various subsets, for example, Th17, Th2, and T_{reg} (for review see (42)). We would speculate that a portion of high avidity effector cells encounter signals subsequent to activation that result in changes which ultimately decrease avidity. As there is the potential for T cells to interact with multiple APC over the first days of activation (19), it is possible that signals delivered by individual APC provide distinct signals that promote modulation. Alternatively, other cells that enter the lymph node over the course of the response may provide modulatory signals.

The question then arises as to what these signals might be. Antigen dose has been established as a potent signal that can drive alteration in avidity (3,16,18,36,37). Thus encounter with APC bearing high pMHC *in vivo* is one potential trigger for reduced avidity. That said, the modulatory APC is unlikely to be a mature DC, since our previous studies suggest that these cells do not induce lower avidity even when presenting high levels of peptide (38). Alternatively, a number of cytokine and co-stimulatory signals have been reported to regulate avidity. For example, concurrent increases in the level of CD80, LFA-3, and ICAM-1 result in generation of a population with higher avidity (45). There are also data to support a role for

inflammatory cytokines (e.g., IL-12 and type I IFN) in increasing CD8⁺ effector cell sensitivity to peptide antigen (53,59,60). In contrast, some cytokine signals (i.e., IL-4) have been reported to decrease pMHC sensitivity (7,50). Inflammatory signals present at high levels early during generation of the response may promote high peptide sensitivity that is subsequently lost in a subset of effectors as the local environment changes over the course of infection.

Our studies are consistent with a model wherein modulation of lck expression contributes to the decreased functional avidity observed in activated effectors present at the peak of the response as compared to those initially responding following infection. Data to support this comes from the increased sensitivity of d6 effector cells to inhibition by lck inhibitors. Interestingly, the disparity in protein level was not reflected in the amount of mRNA present, suggesting that regulation of gene transcription and or mRNA turnover does not differ in these two populations. We would favor the model that the increased amount of lck present in d2 effectors reflects an increased level at steady state, perhaps due to reduced turnover of the protein.

Our studies demonstrate reduced avidity within a defined subset of responding effectors. While this is the first report of an active decrease in avidity in functional effector cells that is associated with changes in lck expression, a previous study reported an increase in the level of lck that correlated with the amount of IFN γ produced by individual cells and with increased sensitivity at the population level (56). In contrast, in a model of intraperitoneal LCMV infection, increases observed in functional avidity were independent of the level of lck (51,53). Interestingly, lck did appear to play a role in the avidity setpoint as reduced peptide sensitivity was associated with increased sensitivity to src kinase inhibitors (51). Thus, the role of lck in controlling avidity appears to be complex, occurring via multiple mechanisms. Further studies are required to elucidate the details of these mechanisms and the circumstances under which they are used (i.e., site of activation, nature of the pathogen).

The generation of lower avidity cells at all may seem at odds with studies that have demonstrated the superior efficacy of high avidity cells for viral and tumor clearance (3,5,6,22,28,34,43,55,61,63). We would speculate that this process may allow for the generation of some breadth in the avidity repertoire as a protective mechanism against supraoptimal stimulation mediated death of the population (4). It is important to note that the avidity repertoire is a spectrum, and while the lowest avidity cells may be of limited usefulness, intermediate avidity cells are likely to contribute to clearance through lysis of infected cells. In addition, these cells would release IFN γ that can promote increased class I expression as well as exert direct anti-viral effects. The generation of lower avidity cells may serve as a backup in the event that pMHC levels in the periphery are substantially higher than in the lymph node. One such scenario is that the infection of the activating APC may be abortive, resulting in limited presentation and a repertoire biased toward high avidity. Encounter of high avidity cells with a highly permissive, high pMHC presenting cell in the periphery could promote death as a result of overstimulation (2,4,64). Intermediate/lower avidity cells would be resistant to this effect.

In summary, our data support a model of active downward tuning of avidity *in vivo* within a subset of the responding CD8⁺ T cells during the anti-viral immune response. This occurs in the endogenous polyclonal population in the absence of any constraints imposed by artificially high precursor frequencies or TCR restrictions. Avidity modulation allows for the generation of an effector pool with a breadth of avidity, which may be beneficial for ensuring a repertoire that contains cells that are optimally tuned for recognition of the level of peptide presented in the infected tissue.

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Author Disclosure Statement

The authors have no conflicts of interest.

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